EXHIBIT A

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Down-regulated Peroxisome Proliferator-activated Receptor γ (PPARγ) in Lung Epithelial Cells Promotes a PPARγ Agonist-reversible Proinflammatory Phenotype in Chronic Obstructive Pulmonary Disease (COPD)*[S]

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Background: The mechanistic role of peroxisome proliferator-activated receptor γ (PPARγ) in chronic obstructive pulmonary disease (COPD) is poorly understood.
Results: COPD and cigarette smoke exposure down-regulated PPARγ and produced inflammation that PPARγ agonists reversed through multiple pathways.
Conclusion: PPARγ plays a pivotal role in COPD.
Significance: PPARγ agonists may be the first effective treatment for COPD.

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory condition and a leading cause of death, with no available cure. We assessed the actions in pulmonary epithelial cells of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor with anti-inflammatory effects, whose role in COPD is largely unknown. We found that PPARγ was down-regulated in lung tissue and epithelial cells of COPD patients, via both reduced expression and phosphorylation-mediated inhibition, whereas pro-inflammatory nuclear factor-κB (NF-κB) activity was increased. Cigarette smoking is the main risk factor for COPD, and exposing airway epithelial cells to cigarette smoke extract (CSE) likewise down-regulated PPARγ and activated NF-κB. CSE also down-regulated and post-translationally inhibited the glucocorticoid receptor (GR-α) and histone deacetylase 2 (HDAC2), a corepressor important for glucocorticoid action and whose down-regulation is thought to cause glucocorticoid insensitivity in COPD. Treating epithelial cells with synthetic (rosiglitazone) or endogenous (10-nitro-oleic acid) PPARγ agonists strongly up-regulated PPARγ expression and activity, suppressed CSE-induced production and secretion of inflammatory cytokines, and reversed its activation of NF-κB by inhibiting the IκB kinase pathway and by promoting direct inhibitory binding of PPARγ to NF-κB. In contrast, PPARγ knockdown via siRNA augmented CSE-induced chemokine release and decreases in HDAC activity, suggesting a potential anti-inflammatory role of endogenous PPARγ. The results imply that down-regulation of pulmonary epithelial PPARγ by cigarette smoke promotes inflammatory pathways and diminishes glucocorticoid responsiveness, thereby contributing to COPD pathogenesis, and further suggest that PPARγ agonists may be useful for COPD treatment.

Chronic obstructive pulmonary disease (COPD)[2] is a progressive disease that, due to lack of effective treatment (1), is a leading cause of death in the United States and worldwide. It is characterized by chronic pulmonary inflammation and long term tissue destruction that impairs respiratory gas exchange. The major risk factor for COPD is exposure to cigarette smoke, which contains noxious inflammatory and oxidant agents. Once established, COPD continues to progress even with smoking cessation or available treatments.

The mainstay of treatment for most inflammatory diseases is glucocorticoid therapy, but in COPD patients it provides only short term benefit (2). Recent studies have attributed such glucocorticoid ineffectiveness to decreased activity of the corepressor histone deacetylase 2 (HDAC2) (3), an essential component of a major mechanism of glucocorticoid action. Oxidative stress (4, 5) and cigarette smoke (6) reduce HDAC2 levels in airway epithelial cells, thereby impairing the anti-inflammatory effectiveness of glucocorticoid receptor (GR-α) activation.

The ligand-activated transcription factor peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor superfamily, exerts strong anti-inflammatory and antioxidant effects (7, 8) by down-regulating activity of nuclear factor-κB (NF-κB) and other pro-inflammatory transcription factors via multiple mechanisms. These actions might be pathophysiologically or therapeutically relevant to COPD, but the potential roles of PPARγ and its agonists in responses to

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[2] The abbreviations used are: COPD, chronic obstructive pulmonary disease; HDAC2, histone deacetylase 2; PPARγ, peroxisome proliferator-activated receptor γ; GR-α, glucocorticoid receptor; HBE, human bronchial epithelial; CSE, cigarette smoke extract; Rosi, rosiglitazone; OA-NO_2, 10-nitro-oleic acid; IKK, IκB kinase; ROS, reactive oxygen species; p, phosphorylated.

cigarette smoke exposure and COPD have previously been poorly characterized. Known PPARγ agonists include the synthetic thiazolidinediones, used to treat type 2 diabetes, and various endogenous compounds. Physiologically relevant endogenous PPARγ agonists remain to be identified, but plausible candidates include nitrated fatty acids, which constitute one of the largest blood-borne pools of biologically active nitrogen compounds (9) and circulate in concentrations sufficient to activate PPARγ (10).

Here we assessed the anti-inflammatory potential of PPARγ in pulmonary epithelial cells of people with and without COPD and on smoke-induced epithelial responses. We also explored its mechanistic relationships with key transcription/signaling factors including the NF-κB pathway, GR-α, and HDAC2. We found that PPARγ expression and activity are down-regulated in human bronchial epithelial (HBE) cells from COPD patients and those exposed to cigarette smoke extract (CSE) *in vitro*, whereas proinflammatory pathways are up-regulated. Treating lung epithelial cells with either the thiazolidinedione rosiglitazone (Rosi) or the endogenous PPARγ agonist 10-nitro-oleic acid (OA-NO_2) reversed these CSE effects and the accompanying decreases in GR-α and HDAC2. PPARγ agonists also blocked CSE-induced inflammatory cytokine and chemokine production and ROS production by reversing the CSE-induced increase in NF-κB activity through multiple PPARγ-mediated mechanisms. Conversely, PPARγ knockdown augmented CSE responses. These findings raise the possibility that PPARγ agonists may be therapeutically useful for treating COPD, and furthermore, may reverse COPD patients' resistance to anti-inflammatory steroid therapy by restoring impaired HDAC2 activity.

EXPERIMENTAL PROCEDURES

Cells—H292 cells were obtained from the ATCC (Rockville, MD) and maintained in RPMI medium supplemented with 10% FBS, 10,000 units/ml penicillin, and 10,000 μg/ml streptomycin (HyClone, Logan, UT). Normal human bronchial epithelial cells and diseased human bronchial epithelial (COPD) cells were obtained from Lonza (Walkersville, MD) at passage 1 and used at passages 2–8. Cells were grown and maintained in bronchial epithelial cell growth medium (BEGM), supplemented with 10% FBS, 0.4% bovine pituitary extract, 0.1% insulin, 0.1% human EGF, 0.1% hydrocortisone, 0.1% GA-1000, 0.1% retinoic acid, 0.1% transferrin, 0.1% triiodothyronine, epinephrine, penicillin, and streptomycin (HyClone). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2, 95% air in tissue culture flasks, plates, or dishes. Monolayer cultures at 90% confluence were deprived of serum for 24 h prior to treatment.

Patient Lung Tissue Samples—Human lung tissues were obtained from excess pathologic tissue after lung transplantation and organ donation, under a protocol approved by the University of Pittsburgh Institutional Review Board. COPD lung tissues were obtained from explanted lungs of subjects with advanced COPD, and control lungs were donated lungs not suitable for transplantation from the Center for Organ Recovery and Education (CORE). Lung tissues were stored at −80 °C until future usage.

Preparation of Cigarette Smoke Extract—CSE was prepared by slowly bubbling smoke from one research-grade cigarette

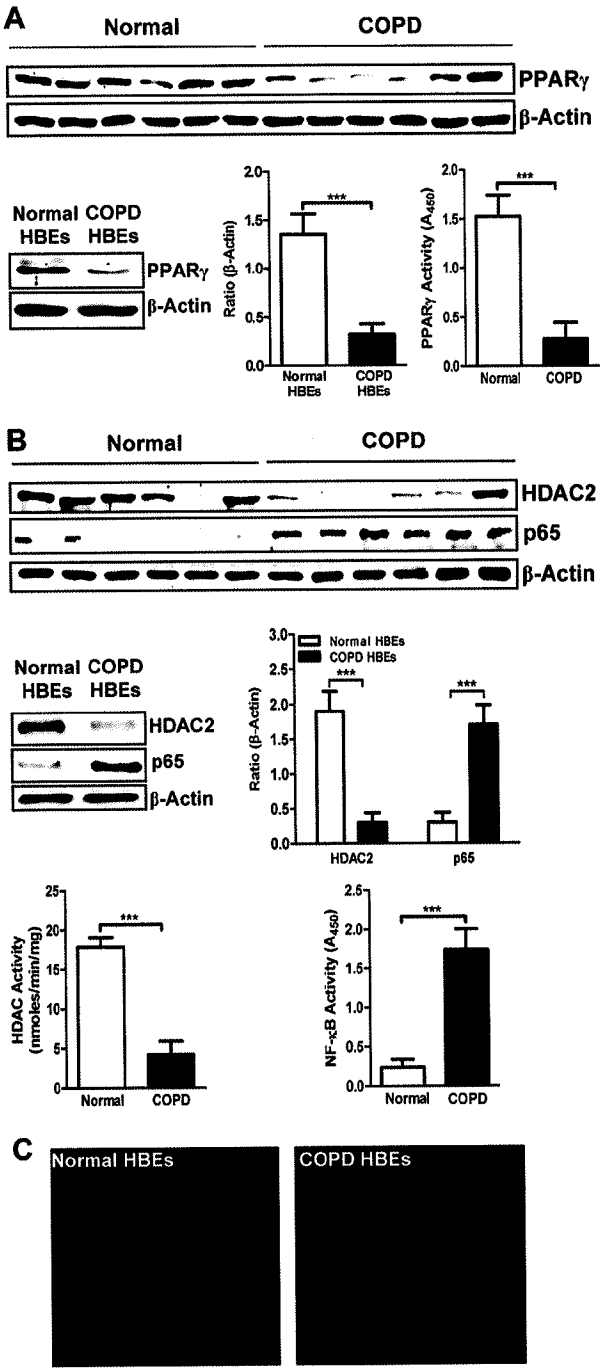

FIGURE 1. **PPARγ expression and activity are decreased in COPD.** *A*, Western blots for PPARγ in tissue extracts of pathologically normal (non-COPD; $n = 6$) and COPD ($n = 6$) lung (*top panel*) and in whole-cell extracts of HBE cells obtained from normal ($n = 6$) and COPD ($n = 6$) subjects (*bottom left panel*) followed by densitometric analysis (*bottom center panel*). *Bottom right panel*, DNA binding activity of PPARγ in non-COPD and COPD tissue extracts measured using an ELISA-based assay. *B*, Western blots and densitometric analysis for HDAC2 and p65 from lung (*top panel*) and HBE cells (*middle left panel*) as described in *A*. *Bottom left panel* and *bottom right panel*, HDAC deacetylase (*bottom left panel*) and p65 DNA binding activities (*bottom right panel*) measured by ELISA-based assays, as described in *A*. *C*, intracellular ROS levels in normal and COPD HBE cells, assessed by confocal microscopy. Data are representative of three independent experiments; ***, $p < 0.001$.

(3R4F; Kentucky Tobacco Research and Development Centre, University of Kentucky, Lexington, KY) into 10 ml of medium according to the Federal Trade Commission (FTC) protocol,

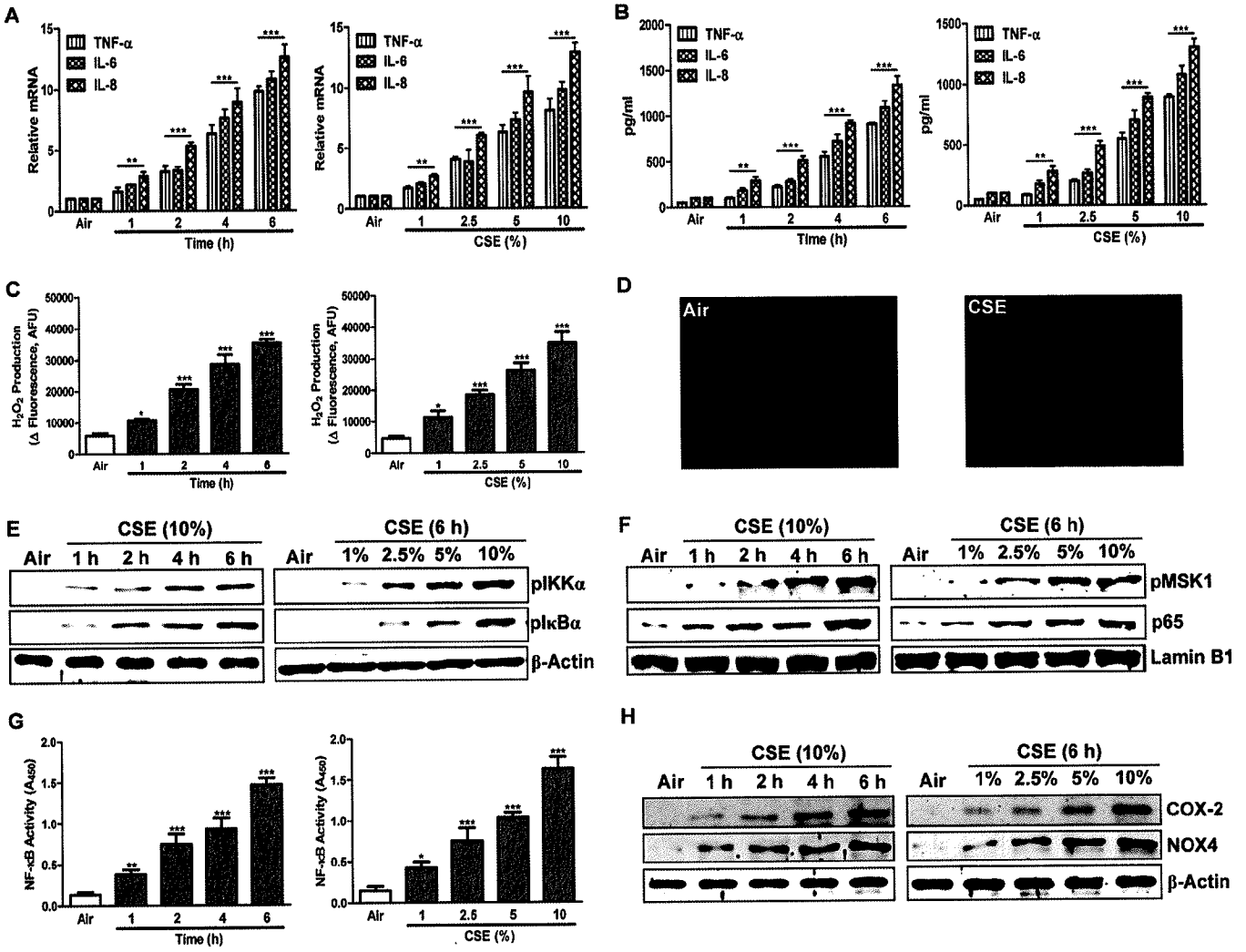

FIGURE 2. **CSE induces inflammatory responses and oxidative stress via up-regulation of NF-κB.** *A* and *B*, shown are time courses and concentration-response relationships for CSE-induced cytokine/chemokine gene expression (*A*) and release (*B*). *C*, H_2O_2 production. *G*, DNA binding activity of NF-κB p65, measured by ELISA-based assay. *E*, *F*, and *H*, p-IKKα and p-IκBα (*E*), p-MSK1 and NF-κB p65 (*F*), and COX-2 and NOX4 protein levels (*H*), by Western blots. *D* shows ROS immunofluorescence in H292 cells exposed for 6 h to 10% CSE or air control. H292 cells were treated with various concentrations (1–10%) of CSE for 6 h or for 1–6 h with 10% CSE, as indicated. In *A*, RNA was isolated, and gene expression levels were analyzed by real-time PCR. *B* and *C* show levels measured in culture medium. *E* and *H*, in whole-cell extracts; *F* and *G*, in nuclear extracts. Data are representative of three independent experiments, with $n = 3$. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

each puff being of 2-s duration and 35-ml volume. The pH of CSE was adjusted to 7.4 and sterilized by filtration through a 0.22-μm filter (EMD Millipore, Billerica, MA). The extract, defined as 100% CSE, was diluted to the indicated concentrations and used within 10 min of preparation. For control experiments, air was bubbled into 10 ml of medium, which was then treated as for CSE.

Measurement of Cytokine and Chemokine Levels in Culture Medium—Cell culture medium from different treatment groups was collected and stored at −80 °C. Levels of TNF-α, IL-6, and IL-8 were measured using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Determination of Cellular ROS—H_2O_2 production in cell culture media was determined using the Amplex Red hydrogen peroxide assay kit (Molecular Probes, Eugene, OR). Production of intracellular ROS in live cells was determined using the Cell Meter fluorimetric intracellular total ROS activity assay kit (AAT Bioquest, Sunnyvale, CA). Samples were mounted on glass slides with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). The slides were viewed by an Olympus FluoView FV1000 confocal microscope (Olympus, Center Valley, PA) using a 60 × fluorescence lens along with FluoView confocal software (FV10-ASW v1.7, Olympus).

HDAC Activity and Transcription Factor DNA Binding Activity Assays—Nuclear proteins were extracted using a nuclear extraction kit (Active Motif, Carlsbad, CA), and their concentrations were determined using the BCA protein assay kit (Pierce). Nuclear extracts were used to quantify HDAC activity and DNA binding activity of PPARγ, GR-α, and the p65 subunit of NF-κB using ELISA-based kits (56210, 40196, 45496, and 40096; Active Motif).

Western Blotting—Total protein extracts were prepared, and Western blotting was performed as described previously (11). Antibodies against PPARγ, p65, COX-2, NOX4, GR-α, HDAC2, p300, β-actin, and lamin B1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p-Ser,

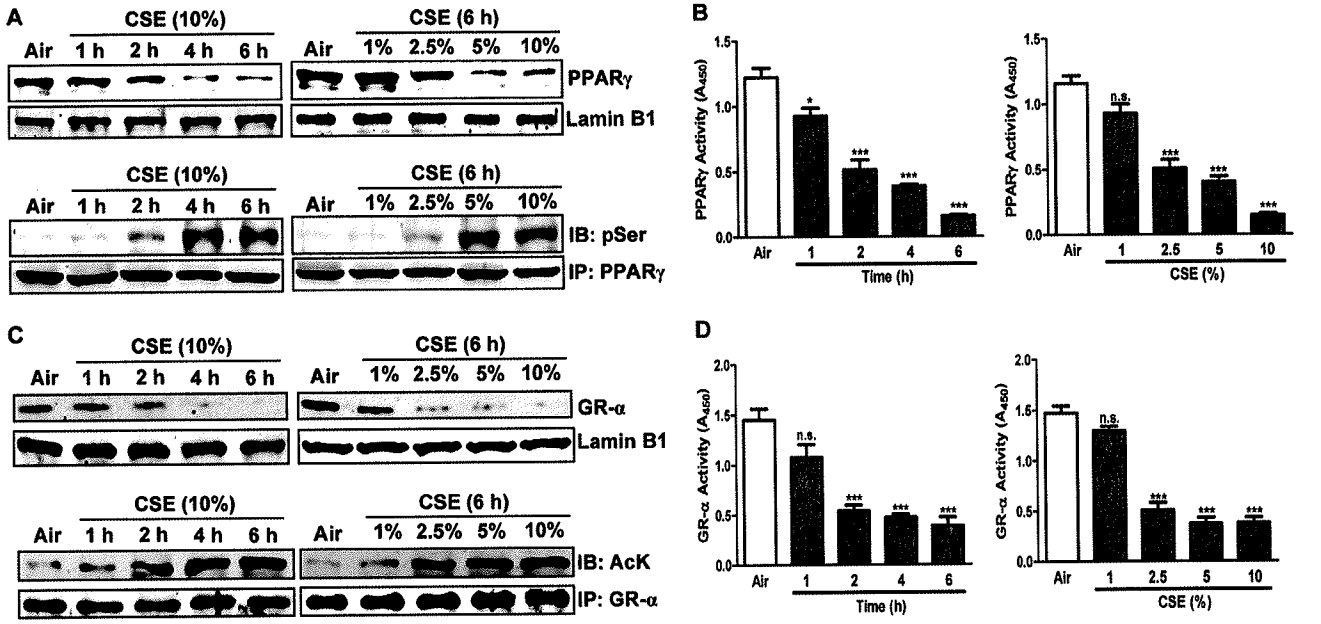

FIGURE 3. **CSE down-regulates anti-inflammatory transcription factors PPARγ and GR-α.** Time courses and concentration-response relationships of transcription factor nuclear localization, modification, and activity in CSE-treated H292 cells. *A*, Western blots (*IB*) of nuclear PPARγ (*top panel*) and phosphorylation of immunoprecipitated (*IP*) PPARγ (*bottom panel*). *B*, PPARγ DNA binding activity, measured using an ELISA-based assay. *C*, Western blots of nuclear GR-α (*top panel*) and acetylation of immunoprecipitated GR-α (*bottom panel*). *D*, GR-α DNA binding activity measured using an ELISA-based assay. H292 cells were treated with various concentrations (1–10%) of CSE for 6 h or for 1–6 h with 10% CSE, as indicated. Data are representative of three independent experiments, with $n = 3$–5. *, $p < 0.05$, ***, $p < 0.001$, *n.s.* = nonsignificant.

p-IKKα, p-IκBα, p-MSK1, acetylated lysine (Ack), acetyl- and phospho-histone H3 (Lys-9/Ser-10), acetyl-histone H4 (Lys-12), histone H3, and histone H4 were from Cell Signaling Technology (Beverly, MA). Antibody against 4-hydroxy-2-nonenal was from Oxis International, Inc. (Beverly Hills, CA). Antibody against nitrotyrosine was from EMD Millipore. Following primary antibody reaction, the membrane was washed in Tris-buffered saline with Tween 20 (TBST) and incubated with a 1:5000 dilution of secondary antibodies consisting of donkey anti-mouse IR-680 (red) and goat anti-rabbit IR-780 (green), both from LI-COR Biosciences (Lincoln, NE), for 1 h at room temperature. The infrared signal was detected using an Odyssey infrared imager (LI-COR).

Immunoprecipitation—Nuclear extracts were prepared and were immunoprecipitated using the Dynabeads protein G immunoprecipitation kit (Invitrogen). Antibodies were bound to Dynabeads protein G, and Dynabeads-antibody complex was used to precipitate target proteins from the nuclear extracts. Unbound proteins were washed away, and complexes were eluted. All samples (20 μg/lane) were separated by electrophoresis on SDS-polyacrylamide gels and transferred to PVDF membranes, and Western blotting was performed.

ChIP Assay—The ChIP assay was performed using the SimpleChIP enzymatic chromatin immunoprecipitation kit with magnetic beads (Cell Signaling Technology). Briefly, cellular chromatin was cross-linked with 1% formaldehyde for 10 min at room temperature, the cross-linking was stopped with 0.125 M glycine, and cells were washed twice with ice-cold PBS. Nuclei were pelleted and digested by micrococcal nuclease. Following sonication and centrifugation, equal amounts of sheared chromatin were incubated overnight at 4 °C with antibodies, IgG as negative control, and RNA polymerase II as positive control (Cell Signaling Technology). Protein G magnetic beads were then added, and the chromatin was incubated with rotation for 2 h at 4 °C. An aliquot of chromatin that was not incubated with any antibody was used as the input control sample. Antibody-bound protein-DNA complexes were eluted and subjected to real-time PCR as described under "RNA Isolation and Quantitative Real-time RT-PCR" with specific primers for TNF-α, IL-6, IL-8, and α-satellite (supplemental Table 1).

RNA Isolation and Quantitative Real-time RT-PCR—RNA was isolated using the RNeasy mini kit (Qiagen), and cDNA was generated from 100 ng of total RNA using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) employing random and oligo(dT) primers. Real-time quantitative PCR was performed using 100 ng of cDNA with 2× SYBR Green master mix (Applied Biosystems) and specific primers for the genes of interest (supplemental Table 1). These experiments were performed on an AB 7500 fast thermal cycler using a three-step protocol employing the melting curve method. The average of each gene cycle threshold (C_t) was determined for each experiment. Relative cDNA levels ($2^{-\Delta\Delta C_t}$) for the genes of interest were determined using the comparative C_t method, which generates the $\Delta\Delta C_t$ as the difference between the gene of interest and the housekeeping genes β-actin and 9 S rRNA for each sample. Each averaged experimental gene expression sample was compared with the averaged control sample, which was set to 1.

Transfecting Small Interfering RNA into Normal HBE Cells—Normal HBE cells were incubated for 8 h with a liposome complex containing 100 nM of small interfering RNA (siRNA) targeted to PPARγ or scrambled control (Dharmacon, Lafayette, CO; supplemental Table 2) and Lipofectamine 2000 (Invitrogen) under serum- and antibiotic-free conditions. After 8 h, fresh medium with 10% FBS was added, and the cells were incu-

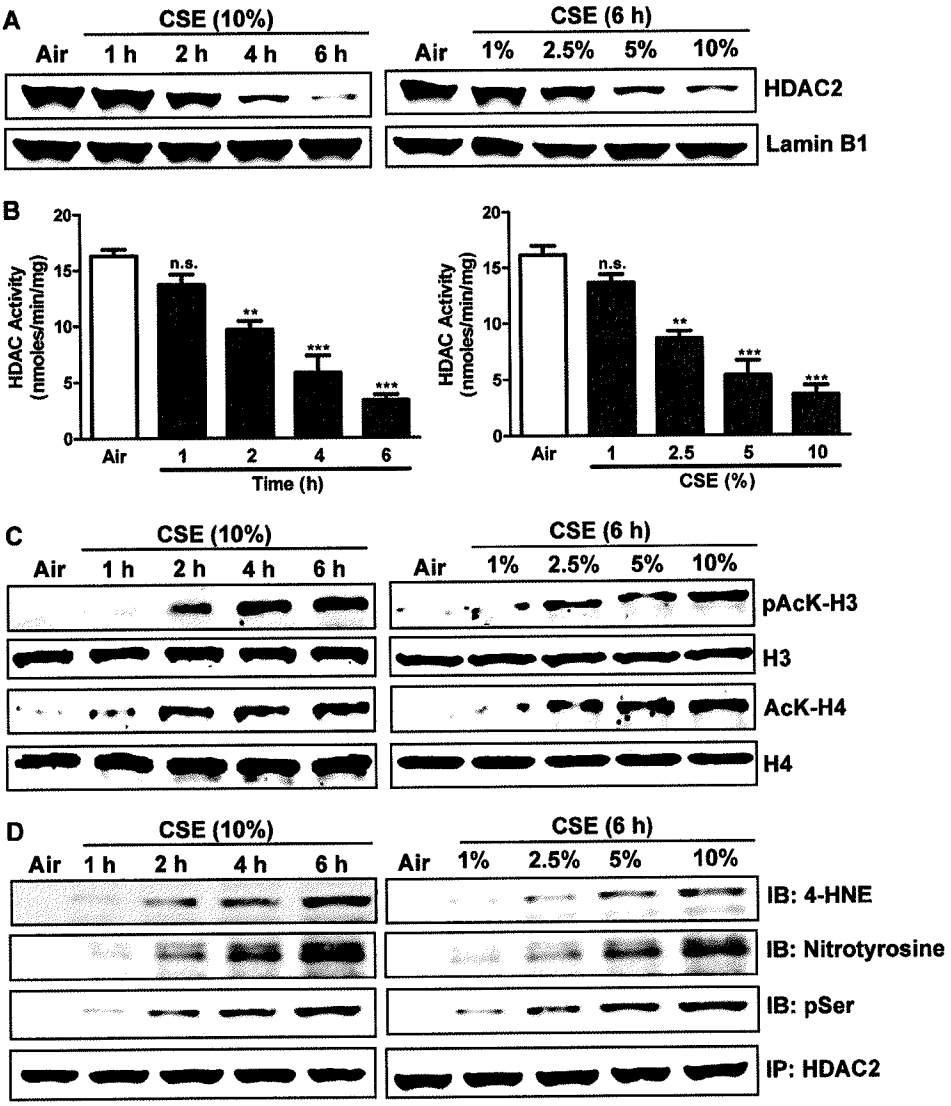

FIGURE 4. **CSE down-regulates HDAC2 and increases chromatin acetylation.** Shown are time courses and concentration-response relationships of effects on HDAC2 and histone acetylation in CSE-treated H292 cells. *A*, Western blots of nuclear HDAC2. *B*, deacetylation activity of HDAC measured using an ELISA-based assay. *C*, Western blots showing acetyl- and phospho-histone H3 (Lys-9/Ser-10) and total histone H3; acetylated (Lys-12) and total histone H4. *D*, Western blots showing immunoprecipitated HDAC2 alkylation with 4-hydroxy-2-nonenal (*4-HNE*), nitrosylation, and phosphorylation. H292 cells were treated with various concentrations (1–10%) of CSE for 6 h or for 1–6 h with 10% CSE, as indicated. Data are representative of three independent experiments, with $n = 3$. *, $p < 0.05$, ***, $p < 0.001$, *n.s.* = nonsignificant.

bated for a further 16 h. After a 24-h incubation, cells were treated with CSE as described.

Statistical Analysis—Data are presented as mean ± S.D. Differences between groups were analyzed using an unpaired *t* test or analysis of variance followed by a Bonferroni's multiple comparison test using GraphPad Prism 5.03 (GraphPad Software, La Jolla, CA). A $p < 0.05$ was considered significant.

RESULTS

PPARγ Down-regulation in COPD Is Associated with Reduction of HDAC2 and Activation of NF-κB—To assess the potential pathophysiological role of PPARγ in COPD, we tested whether PPARγ expression and function are altered in lung tissue samples of COPD patients and in airway epithelial cells. These cells are directly smoke-exposed in cigarette smokers and are pathogenic targets and mediators in COPD (12, 13). We found that PPARγ protein (shown by Western blots) and DNA binding activity (Fig. 1*A*) were reduced in lung tissue and HBE cells of COPD patients when compared with those from individuals without COPD. This PPARγ down-regulation was associated with the previously reported (3) up-regulated expression and activity of the pro-inflammatory transcription factor NF-κB and down-regulation of the corepressor HDAC2 (Fig. 1*B*), a factor that participates in GR-α-mediated anti-inflammatory activity. HBE cells from COPD patients also exhibited oxidative stress consistent with their proinflammatory state, as shown by immunostaining for ROS followed by confocal microscopy (Fig. 1*C*).

CSE Induces Inflammatory Responses and Oxidative Stress in Human Epithelial Cells—Cigarette smoking, the major risk factor for COPD, produces lung inflammation and oxidative stress. To assess the mechanisms by which smoke down-regu-

lates epithelial PPARγ and induces inflammation, we determined the time courses and concentration-response relationships of CSE-induced proinflammatory proteins, transcriptional mediators, and ROS in H292 human lung epithelial cells. Treating cells with varying concentrations of CSE for 6 h or with 10% CSE for various times up-regulated expression and release of the inflammatory cytokines TNF-α and IL-6 and the chemokine IL-8 (Fig. 2, *A* and *B*). Production of H_2O_2 was also increased (Fig. 2*C*) as were intracellular ROS levels seen by immunofluorescence after a 6-h exposure to 10% CSE (Fig. 2*D*). CSE exposure likewise activated the proinflammatory transcription factor NF-κB, seen via increased nuclear NF-κB p65 levels (Fig. 2*F*) and DNA binding activity (Fig. 2*G*), and increased expressed protein levels of its transcriptional targets COX-2 and NADPH oxidase 4 (NOX4) (Fig. 2*H*).

We also measured changes in inhibitor of NF-κB (IκB), which down-regulates activity of NF-κB by preventing its translocation to the nucleus, and in IκB kinase (IKK), which drives ubiquitination and degradation of IκB, thereby increasing NF-κB activity (14). CSE treatment increased the levels of phosphorylated IκB and phosphorylated (activated) IKK (Fig. 2*E*) and those of the activated form of mitogen- and stress-activated protein kinase 1 (MSK1), which phosphorylates IKK (Fig. 2*F*). MSK1 also promotes inflammatory gene transcription by phosphorylating NF-κB itself, allowing it to recruit coactivators, and by phosphorylating histone H3, allowing it to induce chromatin loosening (15). The time courses and concentration-response relationships of all these CSE-induced responses were very similar, pointing to a broadly coordinated proinflammatory program in lung epithelial cells.

CSE Down-regulates PPARγ, GR-α, and HDAC2 while Enhancing Chromatin Acetylation—Based on the CSE-induced proinflammatory profile we saw in H292 cells and the suppression of PPARγ seen in HBE cells of COPD patients, we tested whether CSE influences PPARγ function in H292 cells. CSE induced time- and concentration-dependent decreases in nuclear levels (Fig. 3*A*, *top panel*) and DNA binding activity (Fig. 3*B*) of PPARγ that were accompanied by increased phosphorylation, an inhibitory post-translational modification (Fig. 3*A*, *bottom panel*). To test the idea that CSE-induced down-regulation of GR-α might contribute to the ineffectiveness of glucocorticoids in COPD, we tested the effects of CSE on GR-α. Supporting our hypothesis, exposing H292 cells to CSE down-regulated both expression and activity of GR-α and also induced its lysine acetylation (Fig. 3, *C* and *D*), an inhibitory post-translational modification.

Transcription factors attract coactivators with histone acetyltransferase activity, which acetylates specific lysines in histones H3 and H4 and thereby loosens chromatin structure so as to allow RNA polymerase to bind and initiate transcription. GR-α suppresses proinflammatory gene expression in part by associating with NF-κB and attracting the corepressor HDAC2, which inhibits transcriptional activation by deacetylating histones (16). We tested the influence of CSE on this system. CSE decreased the nuclear localization and activity of HDAC2 (Fig. 4, *A* and *B*) and thus increased the acetylation of histones H3 and H4, in a time- and concentration-dependent manner (Fig. 4*C*). CSE-induced suppression of HDAC activity reflected not

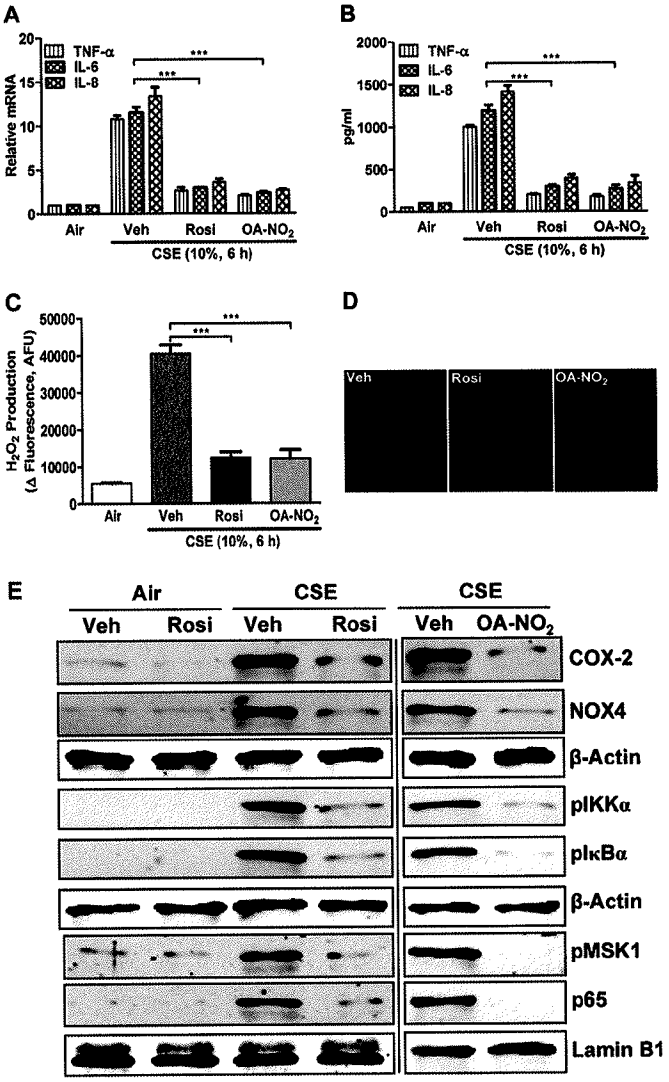

FIGURE 5. **PPARγ activation decreases CSE-induced cytokine production and oxidative stress by modulating NF-κB.** Shown are effects of treatment with Rosi (1 μM) or OA-NO_2 (100 nM) followed by CSE treatment (10%, 6 h) of H292 cells. *A* and *B*, cytokine/chemokine gene expression (*A*) and release (*B*). *Veh*, vehicle. *C*, H_2O_2 production. *D*, intracellular ROS levels. *E*, Western blots showing levels of COX-2, NOX4, p-IKKα, p-IκBα, p-MSK1, and NF-κB p65. In *A*, RNA was isolated, and gene expression levels were analyzed by real-time PCR. *B* and *C* show levels measured in culture medium. *AFU*, arbitrary fluorescence units. *D* was assessed by confocal microscopy. *E*, in whole-cell and nuclear extracts, as indicated by β-actin or lamin B1, respectively. Data are representative of three independent experiments with $n = 3–5$. ***, $p < 0.001$.

only its down-regulated expression, but also induction of multiple concentration- and time-dependent post-translational modifications including serine phosphorylation, tyrosine nitrosylation, and cysteine alkylation by 4-hydroxy-2-nonenal, a specific marker of oxidative stress (Fig. 4*D*).

PPARγ Activation Reduces CSE-induced Inflammation and Oxidative Stress while Up-regulating GR-α and HDAC2—To determine whether PPARγ activation can suppress the CSE-induced proinflammatory profile and resulting oxidative stress in epithelial cells, we treated H292 cells with either the synthetic agonist Rosi (1 μM) or the endogenous agonist OA-NO_2 (100 nM) for the 30 min preceding a 6-h exposure to 10% CSE. Both PPARγ agonists reduced the CSE-induced increases in

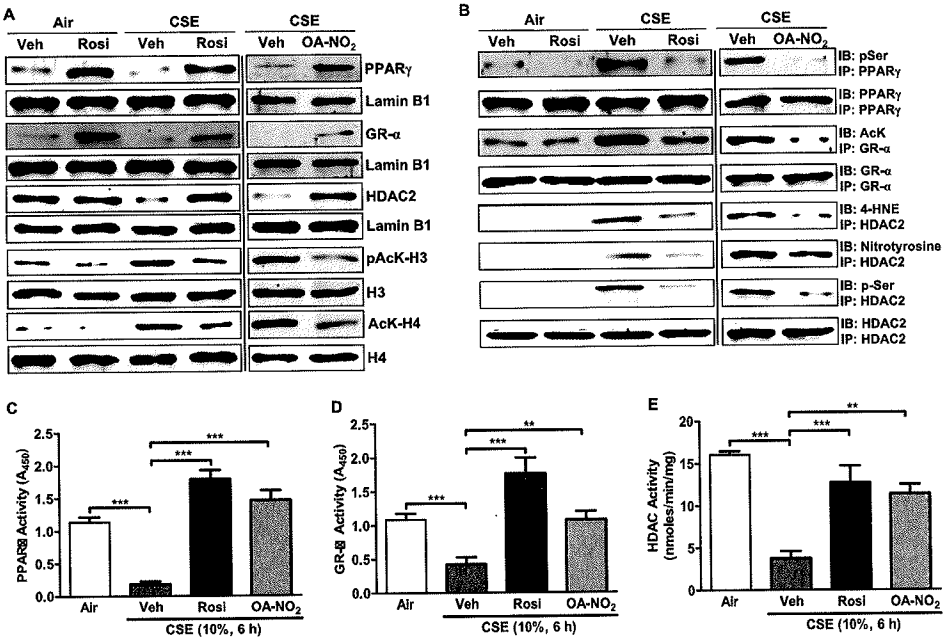

FIGURE 6. **PARγ activation reverses CSE-induced changes in PPARγ, GR-α, and HDAC2 and in chromatin acetylation.** Shown are effects of treatment with Rosi (1 μM) or OA-NO_2 (100 nM) followed by CSE treatment (10%, 6 h) of H292 cells. *A*, Western blots showing nuclear levels of PPARγ, GR-α, and HDAC2, as well as showing acetyl- and phospho-histone H3 (Lys-9/Ser-10) and total histone H3; acetylated (Lys-12) and total histone H4. *Veh*, vehicle. *B*, Western blots (*IB*) showing phosphorylation in immunoprecipitated (*IP*) PPARγ, acetylation in immunoprecipitated GR-α, and 4-hydroxy-2-nonenal (*4-HNE*) alkylation, nitrosylation, and phosphorylation in immunoprecipitated HDAC2. *C*, DNA binding activity of PPARγ. *D*, DNA binding activity of GR-α. *E*, deacetylation activity of HDAC. All activities were measured using ELISA-based assays. Data are representative of three independent experiments with $n = 3$–5. **, $p < 0.01$, ***, $p < 0.001$.

cytokine gene expression (Fig. 5*A*) and secretion (Fig. 5*B*) by ~80% and similarly reduced H_2O_2 production (Fig. 5*C*) and intracellular ROS seen by immunostaining (Fig. 5*D*). Both agonists likewise markedly reduced the levels of NF-κB and its target proteins COX-2 and NOX4, as well as the phosphorylated forms of MSK1, IKK, and IκB (Fig. 5*E*). These data indicate that agonist-induced PPARγ activation inhibits the proinflammatory effects of CSE.

To determine the mechanisms by which PPARγ activation exerts its anti-inflammatory and antioxidant effects, we studied the effects of Rosi or OA-NO_2 on nuclear localization and activity of PPARγ, GR-α, and HDAC. Treatment with Rosi or OA-NO_2 more than reversed the decreases in nuclear PPARγ levels caused by CSE exposure, raising levels even above the air-vehicle treatment baseline (Fig. 6*A*). Both PPARγ agonists likewise up-regulated DNA binding activity of PPARγ and GR-α along with the deacetylase activity of HDAC2 (Fig. 6, *C–E*). These increases in activity were accompanied by decreases in PPARγ phosphorylation, GR-α acetylation, and the levels of HDAC2 phosphorylation, nitrosylation, and alkylation (Fig. 6*B*). The increased HDAC2 activity was associated with decreased acetylation of histones H3 and H4 (Fig. 6*A*). Taken together, these data demonstrate that PPARγ activation can reverse CSE-induced proinflammatory effects and ROS generation in epithelial cells and suggest that this is due both to direct anti-inflammatory effects of PPARγ itself and to restoration of the HDAC2 activity required by GR-α for effective anti-inflammatory action.

Activation of PPARγ Modifies CSE-induced Protein-protein and Protein-DNA Associations—Transcription is regulated both by direct modulation of the activities of relevant transcription factors and their abilities to attract and bind coactivators or corepressors. Ligand-activated PPARγ inhibits NF-κB by direct binding, among other mechanisms (17), while GR-α links NF-κB to the corepressor HDAC2 and blocks its recruitment of the histone acetyltransferase coactivator p300 and the coactivator MSK1. Co-immunoprecipitation experiments showed that CSE exposure greatly increased the amounts of p300 and MSK1 associated with nuclear NF-κB. These effects were largely reversed by treatment with either Rosi or OA-NO_2 (Fig. 7*A*). PPARγ activation likewise reversed the CSE-induced decrease in association between GR-α and NF-κB, while NF-κB was further inhibited by increased direct association with PPARγ. Rosi or OA-NO_2 treatment also increased the association of HDAC2 and of PPARγ with GR-α (Fig. 7*B*). We confirmed by ChIP that the observed agonist-induced diminution of CSE-elevated nuclear localization of NF-κB (Fig. 7*C*) and MSK1 (Fig. 7*D*) was associated with reduced binding to the promoter regions of the NF-κB target genes TNF-α, IL-6, and IL-8. This observation further implies that MSK1 was bound to NF-κB.

PPARγ Knockdown Amplifies and Activation Reverses CSE Effects in HBE cells—To test whether endogenous PPARγ activity suppresses the CSE-induced proinflammatory and related transcriptional program in HBE cells, we used a gene silencing approach. Treating HBE cells with PPARγ-directed siRNA abrogated PPARγ expression to the suppressed levels seen with CSE treatment (Fig. 8*A*), while agonist treatment up-regulated expression in whole-cell extracts above vehicle-treated baseline (Fig. 8*B*). Such PPARγ knockdown augmented CSE-induced alterations in HDAC and NF-κB activity (Fig. 8*C*) and in CSE-induced stimulation of IL-8 release (Fig. 8*D*) *versus* those seen

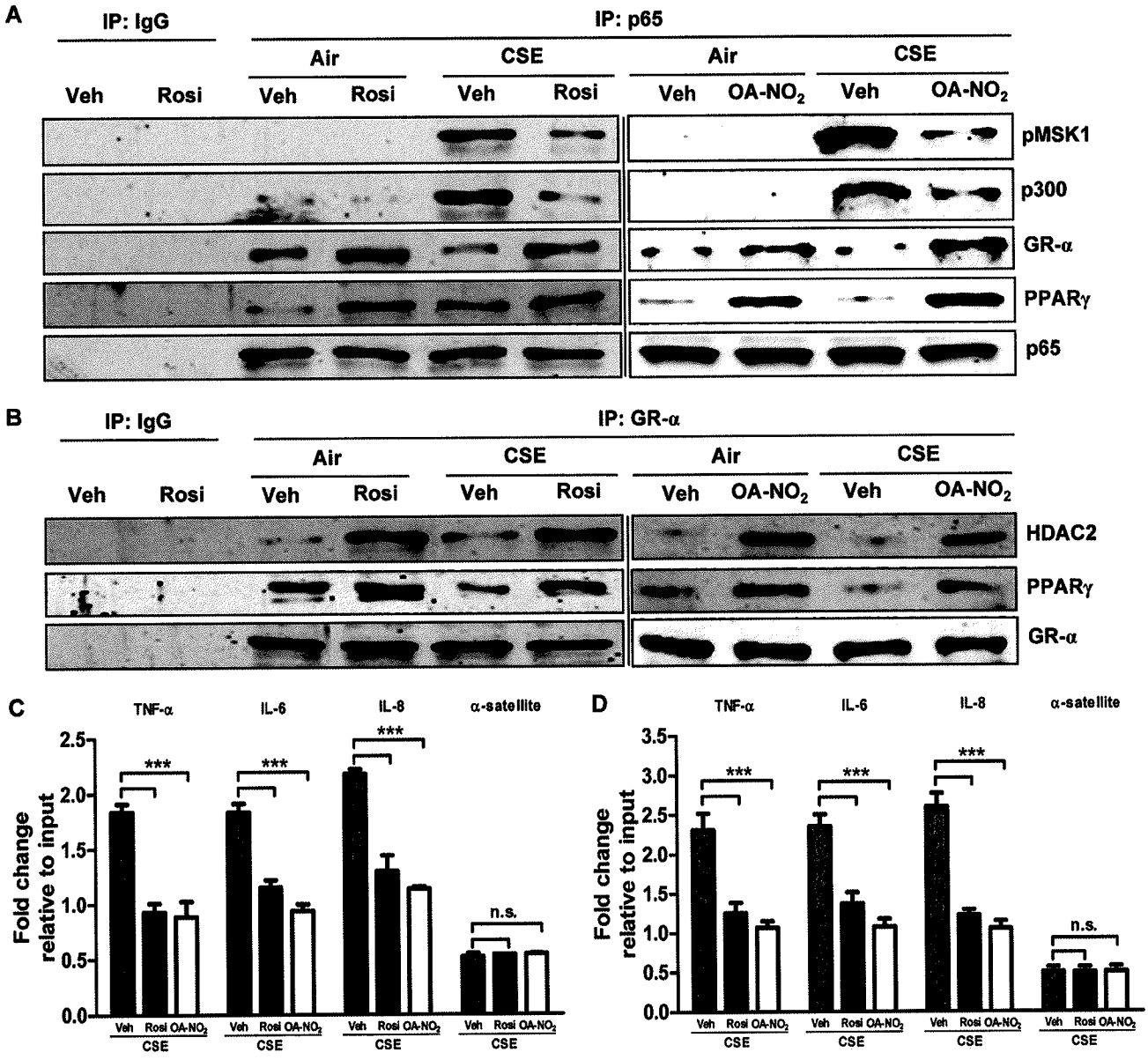

FIGURE 7. **PPARγ activation modulates CSE-induced protein-protein and protein-DNA interactions.** Shown are effects of treatment with Rosi (1 μM) or OA-NO_2 (100 nM) followed by CSE treatment (10%, 6 h) of H292 cells. *A* and *B*, nuclear extracts were immunoprecipitated (*IP*) for NF-κB p65 and GR-α and Western blotted for p-MSK1, p300, GR-α, PPARγ, and p65 (*A*) and HDAC2, PPARγ, and GR-α (*B*). *Veh*, vehicle. *C* and *D*, chromatin was cross-linked and immunoprecipitated with antibodies against p65 (*left*) and p-MSK1 (*right*). Antibody-bound protein-DNA complexes were eluted and subjected to real-time PCR with specific primers for TNF-α, IL-6, IL-8, and α-satellite DNA. Data are representative of three independent experiments with $n = 3$–5. ***, $p < 0.001$, *n.s.* = nonsignificant.

in wild-type cells. Thus, whereas PPARγ activation reversed the deleterious effects of CSE, reducing PPARγ greatly exaggerated them.

To assess the potential relevance of agonist-induced PPARγ activation to the effects of COPD we saw in HBE cells (Fig. 1), we tested the abilities of Rosi- and OA-NO_2-induced PPARγ activation to inhibit CSE responses in HBE cells. As in H292 cells (Figs. 5–7), both agonists abrogated CSE-induced suppression of PPARγ, GR-α, and HDAC2 levels and its up-regulation of NF-κB (Fig. 8*E*). These effects of Rosi and OA-NO_2 were accompanied by reduced NF-κB binding to the promoter region of its target gene IL-8 (Fig. 8*F*) and decreased CSE-stimulated IL-8 release (Fig. 8*G*). These effects in human lung cells thus show that our results are generalizable beyond H292 cells and support their relevance for the smoke-exposed human airway *in vivo*.

DISCUSSION

Our studies lead to two central conclusions. First, PPARγ expression and DNA binding activity are down-regulated in lung tissue samples and bronchial epithelial cells from COPD patients. These observations are linked to down-regulation of GR-α and its associated corepressor HDAC2, whereas the transcription factor NF-κB is up-regulated. The net effect of these findings is the enhanced airway inflammation typically observed in COPD. CSE treatment of human lung epithelial

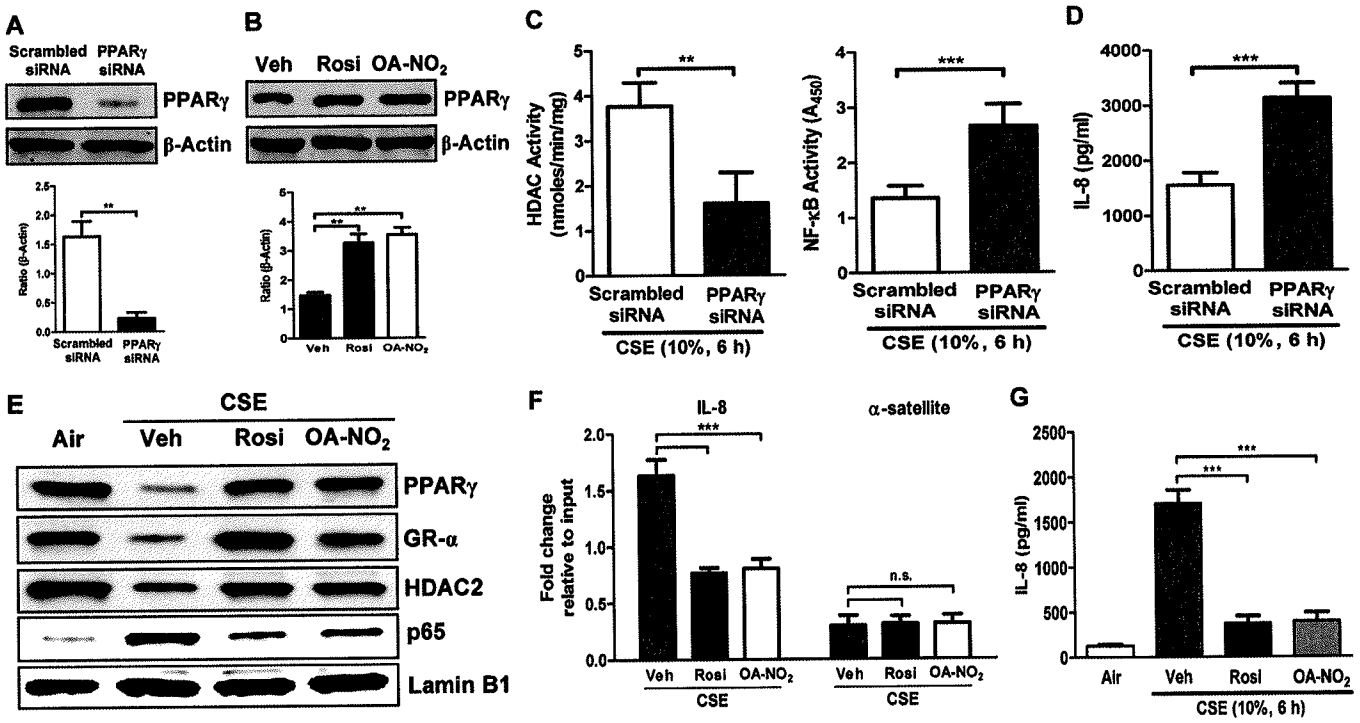

FIGURE 8. **PPARγ knockdown exaggerates whereas PPARγ activation ameliorates CSE-induced inflammatory response in HBE cells.** *A–G*, effects of PPARγ knockdown with siRNA (*A*, *C*, and *D*) and activation with OA-NO_2 (100 nM) or Rosi (1 μM) (*B* and *E–G*) followed by CSE treatment (10%, 6 h) in normal HBE cells. *A* and *B*, Western blots showing whole-cell PPARγ levels following knockdown (*A*) and agonist treatment (without CSE treatment) (*B*). *Veh*, vehicle. *C*, deacetylase activity of HDAC (*left*) and DNA binding activity of NF-κB (*right*) measured using ELISA-based assays. *D*, secretion of IL-8. *E*, Western blots showing nuclear localization of PPARγ, GR-α, HDAC2, and NF-κB p65. *F*, chromatin was cross-linked and immunoprecipitated with antibodies against p65. Antibody-bound protein-DNA complexes were eluted and subjected to real-time PCR with specific primers for IL-8 and α-satellite. *G*, secretion of IL-8. Data are representative of three independent experiments with $n = 3–5$. **, $p < 0.01$, ***, $p < 0.001$, *n.s.* = nonsignificant.

cells recapitulated the effects observed in samples taken directly from COPD patients, and these effects were exaggerated by PPARγ knockdown. Second, these deleterious effects of CSE were reversed by either of two structurally distinct PPARγ agonists, one synthetic, the other endogenous, that each up-regulated expression and activity of the nuclear receptor. These agonists also largely or completely reversed CSE effects on other anti-inflammatory proteins, on the pro-inflammatory transcription factor NF-κB, and on cytokine, chemokine, and ROS production. By demonstrating both PPARγ down-regulation in COPD and the ability of PPARγ activation to reverse all effects in a smoke-induced *in vitro* model of the disease, our findings thus support a crucial role of PPARγ down-regulation in pathogenesis of smoking-induced COPD.

Mechanistic studies supported multiple paths through which PPARγ activation, with consequent increases in its expression and activity, blocks the ability of cigarette smoke to up-regulate NF-κB and thus induce inflammation. Co-immunoprecipitation demonstrated binding of PPARγ to NF-κB, which can inhibit that transcription factor in at least two different ways (17, 18). Furthermore, treatment with either PPARγ agonist reversed the CSE-induced phosphorylation of IKK, thus blocking degradation of IκB and consequent nuclear localization of NF-κB (14). This extends to airway epithelial cells and an additional PPARγ agonist previous observations that the thiazolidinedione pioglitazone blocks IKK activation through a PPARγ-dependent mechanism in IL-1β-stimulated vascular smooth muscle cells (19). The agonist-induced reduction in IKK phosphorylation we see presumably reflects the accompanying decrease in activating phosphorylation of MSK1. The ability of PPARγ to attack inflammation via multiple pathways thus enhances its attractiveness as a potential therapeutic target in COPD.

It is also unclear whether the PPARγ down-regulation and inhibition we observe directly reflect inflammatory signaling, are due to inflammation-associated oxidative stress, or both. Blanquicett *et al.* (20) have reported that H_2O_2 induces a prolonged down-regulation of PPARγ mRNA expression in human vascular endothelial cells. The authors attributed this effect to oxidative stress activation of the transcription factor activator protein 1 (AP1). The inhibitory post-translational phosphorylation we also see appears to reflect a different mechanism, however, since it is driven by activation of mitogen-activated protein kinases (MAPKs). Previous studies have shown that such phosphorylation is mediated by members of the MAPK family (21–24) and that it occurs specifically at Ser-84 (Ser-82 in the mouse) (21, 22). There is redundancy in the pathways involved because PPARγ can be phosphorylated by either extracellular signal-related kinase (21–23) or c-Jun N-terminal kinase (21, 24). Both are members of the MAPK family that can be activated by cigarette smoke (25).

The limited effectiveness of glucocorticoids represents a major therapeutic challenge in COPD. We demonstrated that CSE reduces nuclear localization and activity of both GR-α and the HDAC2 corepressor that it utilizes, with accompanying post-translational modifications of these proteins, and that

Role of PPARγ Down-regulation and Activation in COPD

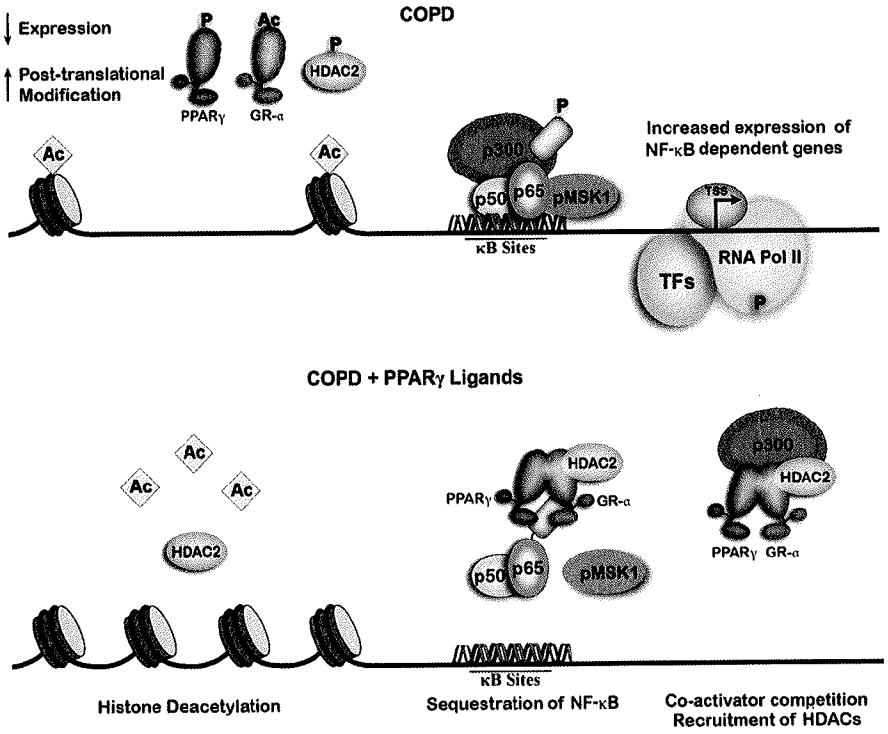

FIGURE 9. **Schematic summary of how PPARγ, GR-α, and HDAC2 affect transcription of NF-κB target genes in COPD.** *Top panel*, cigarette smoke-induced inflammatory signaling, which leads to degradation and post-translational inhibitory modifications of PPARγ, along with HDAC2 and GR-α, results in histone acetylation and chromatin unwinding. This allows NF-κB and its associated coactivators to bind to target promoters and stimulate transcription by RNA polymerase II (*RNA Pol II*). *Bottom panel*, activation of PPARγ up-regulates HDAC2 and GR-α expression in addition to expression of PPARγ itself. This results in removal of histone acetyl groups and condensation of chromatin structure, blocking NF-κB and RNA polymerase II binding. NF-κB activity is further reduced by binding to PPARγ and GR-α and, through GR-α, to HDAC2, as well as by PPARγ competition for essential coactivators.

these effects are reversed by PPARγ activation. This observation suggests that PPARγ activation may restore glucocorticoid sensitivity in lung epithelial cells and potentially in COPD patients, raising the possibility that joint administration of PPARγ and GR-α agonists would be therapeutically appropriate. PPARγ activation also increased its association with GR-α, as has been observed by others (26). Whether this PPARγ-GR-α binding is direct or indirect remains unclear, however. It has been suggested that it may represent binding to common coactivators (26).

A prior study found that PPARγ levels in lung tissues were up-regulated in patients with mild COPD, but in line with our present findings, were down-regulated in those with moderate or severe disease (27). The cell types involved were not identified. Because PPARγ levels were recently found to be unaltered in alveolar macrophages of COPD patients (28), our findings clearly point to lung epithelial cells as a key locus of PPARγ down-regulation and target for its potential therapeutic activation in COPD patients. Other previous investigations have shown that PPARγ activation can attenuate inflammation either in animal models of COPD or following CSE exposure *in vitro*, but have not addressed the signaling pathways we investigated. Both the thiazolidinedione pioglitazone (29) and the endogenous PPARγ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (30) have proven effective in the LPS-induced model of COPD, while both Rosi and pioglitazone were effective in a smoke-induced model (28). *In vitro*, Lee *et al.* (31) attributed the ability of Rosi to inhibit CSE-induced TNF-α and mucin production in H292 cells to up-regulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), with consequent down-regulation of the Akt signaling pathway. However, Rosi did not block CSE-induced cytokine production in a monocyte-macrophage cell line in which CSE disrupts the association between PPARγ and NF-κB (32). Taken together with the previously mentioned study by Lea *et al.* (28), this suggests that the role of PPARγ in COPD may differ between macrophages and epithelial cells.

Our results show that down-regulation of epithelial cell PPARγ expression and activity plays an important role in cigarette smoke-induced inflammation and the pathophysiology of COPD (Fig. 9, *top panel*). Among its other effects, this down-regulation decreases HDAC2 expression and activity, which contributes to increased acetylation of chromatin histones and a looser, unwound chromatin conformation. This conformational change facilitates binding of NF-κB (p50 + p65, expression and activity of which are up-regulated in COPD) and its p300 coactivator to the genes' promoter regions. Acetylation by p300 further loosens chromatic structure, allowing binding of RNA polymerase II and increased transcription of these genes. These changes are reversed by PPARγ activation (Fig. 9, *bottom panel*). This leads to up-regulation of HDAC2 expression and activity, with removal of histone acetyl groups and rewinding of the chromatin. NF-κB, now bound to PPARγ, GR-α, and HDAC2, no longer binds to the promoter regions of its target proteins. Furthermore, activated PPARγ itself attracts the coactivators that NF-κB requires (33), limiting the activity of

any remaining promoter-bound transcription factor. We also show that PPARγ and GR-α simultaneously bound to NF-κB, as suggested by our finding that these two nuclear hormone receptors co-immunoprecipitate.

Our studies thus provide major new insights into the mechanisms by which COPD-induced down-regulation of PPARγ expression and inhibition of its activity contribute to the pro-inflammatory phenotype characteristic of this disease and the ways in which PPARγ agonists reverse these effects. They also illuminate the mechanisms underlying the glucocorticoid insensitivity seen in COPD and imply that PPARγ agonists could restore sensitivity. Taken together, these results support the possibility that PPARγ agonists might prove effective treatments for this common and deadly disease.

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Down-regulated Peroxisome Proliferator-activated Receptor γ (PPARγ) in Lung Epithelial Cells Promotes a PPAR γ Agonist-reversible Proinflammatory Phenotype in Chronic Obstructive Pulmonary Disease (COPD)

Sowmya P. Lakshmi, Aravind T. Reddy, Yingze Zhang, Frank C. Sciurba, Rama K. Mallampalli, Steven R. Duncan and Raju C. Reddy

J. Biol. Chem. 2014, 289:6383-6393.
doi: 10.1074/jbc.M113.536805 originally published online December 24, 2013

EXHIBIT B

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August 10, 2018

VIA ELECTRONIC MAIL
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11200 Rockville Pike
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Re: Journal of Biological Chemistry Publication
Lakshmi, et al. 2014 Article

Dear Dr. Sakabe:

We represent Mr. Aravind Reddy Tarugu, a co-author of Lakshmi SP, Reddy AT, Zhang Y, Sciurba FC, Mallampalli RK, Duncan SR, and Reddy RC, *The Journal of Biological Chemistry* 289(10):6383-6393 (2014), "Down-regulated peroxisome proliferator-activated receptor γ (PPARγ) in lung epithelial cells promotes a PPARγ agonist-reversible phenotype in chronic obstructive pulmonary disease (COPD)" (the "Paper"). Mr. Tarugu was notified in January 2016 of an allegation that "the left band in the left panel in the β-actin plot appears to be the same as the left band in the right panel of the pMSK1 blot, flipped by 180°." A joint inquiry on this matter by the University of Pittsburgh (the "University") and the Department of Veterans Affairs ("VA"), led by the University of Pittsburgh, resulted in dismissal of this matter on February 2016. In a follow up by the VA to this dismissal, the VA requested that the University initiate a joint investigation by the University and the VA led by the University, which resulted in a finding that Mr. Reddy falsified Figure 5E.

After receiving the final investigation report, the University and the VA made separate determinations to which we appealed. After the appeals, the University reversed its decision while the VA affirmed the findings of the University investigation report. Dr. Steven Graham, the Research Integrity Officer for the VA, implemented a corrective action plan for Mr. Reddy which requires him to notify JBC not later than August 10, 2018 of the VA's finding against him. This letter constitutes that notice.

Notwithstanding the VA's finding, Mr. Reddy submits that no action should be taken by JBC with regard to the Paper. With all due respect to the VA, its conclusion that the left band in the right panel of the pMSK1 blot was falsified is wrong and without any preponderance of evidence. In fact, in direct contrast to the VA's decision, the University (the lead institute that

conducted the investigation), after an appeal to the Provost and a review by an Appeal Panel consisting of five Professors, ***unanimously reversed*** the finding against Mr. Reddy that had been made by the joint investigation committee. *See* Attachment No. 1 (redacted copy of Provost Patricia Beeson's April 4, 2018 final decision after appeal) and Attachment No. 2 (redacted copy of the March 27, 2018 recommendation of the University's appeal panel). The Appeal Panel's recommendation for reversing states in pertinent part:

> There was disagreement between the two experts who examined the image evidence [as to whether the bands are identical], and that disagreement was not fully addressed and resolved by the Investigative Board. . . [T]he figure panel at the center of the investigation was not essential for the paper's conclusions.
>
> A finding of no misconduct on your part will be reported.

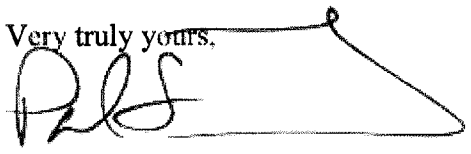
Attachment No. 2 at p.3. In reversing, Provost Beeson expressly withdrew the sanction issued by Assistant Dean Saleem Khan (following the investigation) which would have required Mr. Reddy to retract the Paper – the same sanction being upheld by VA.

The decision of Provost Beeson and the recommendation of the Appeal Panel reversing the finding is supported by two experts in the field, Dr. Alan Price and Dr. John Dahlberg whose opinions are attached as Attachment Nos. 3 and 4, respectively. Dr. Price spent 17 years working with ORI, having served in the latter years as the Associate Director for Investigative Oversight for ORI. During that time, his group was involved with over 3,000 allegations/queries and over 700 formal inquiries or investigations, and they made more than 175 findings of scientific/research misconduct. During his last decade in ORI, Dr. Price reviewed and handled all of the allegations received by ORI from individuals, institutions, and the National Institutes of Health (NIH). Dr. Dahlberg spent 23 years at ORI. In 2006, he became the Director of the Division of Investigational Oversight (DIO), the division within ORI responsible for conducting oversight review of institutional inquiries and investigations involving questioned research funded by the Public Health Service. In mid-2013, he became Deputy Director of ORI where he remained until his retirement in April 2015. During his tenure at ORI, Dr. Dahlberg handled thousands of allegations and dealt with hundreds of cases. Both Dr. Price and Dr. Dahlberg have reviewed the pertinent documents and data, and they agree that a preponderance of the evidence does not establish that the two images are identical. They agree with the University's decision to reverse the finding against Mr. Reddy.

The VA stands alone in its conclusion that the image in Figure 5E was falsified without exhibiting any preponderance of evidence. Neither Provost Beeson, the University's Appeal Panel, Dr. Price or Dr. Dahlberg agree. While we have brought the VA's decision to your attention as directed by Dr. Graham, we respectfully submit that the VA's decision does not warrant that any action be taken with respect to the Paper.

Should you or the Publications Committee have any questions or need further information, please do not hesitate to contact me.

Very truly yours,



Paul S. Thaler

cc: (via email)
Mr. Aravind Reddy Tarugu
Dr. Steven Graham

ATTACHMENT 1

University of Pittsburgh

Office of the Provost and Senior Vice Chancellor

801 Cathedral of Learning
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Fax: 412 3839840
beeson@pitt.edu

Patricia E. Beeson
Provost
Senior Vice Chancellor

April 4, 2018

Aravind Tarugu
Senior Research Associate
Department of Medicine
Montefiore NW 628

Dear Mr. Tarugu:

In response to your appeal from the decisions of School of Medicine Assistant Dean for Faculty Affairs Saleem Khan following his receipt of the report of the Investigative Board, an Appeal Panel was appointed. Your appeal requested review of the research misconduct finding [redacted]. I have reviewed the Panel's recommendations sent to me in late March, which I am attaching to this letter.

My decision is to accept the recommendation of the Panel that Dr. Khan's finding of research misconduct on your part be reversed [redacted].

[redacted]

Public Health Service Policy requires the University to report the result of the investigation to the Office of Research Integrity, US Department of Health and Human Services. A finding of no research misconduct on your part will be reported.

[redacted] Therefore this decision shall be the final disposition of this matter on behalf of the University.

Sincerely,

Patricia E. Beeson

ATTACHMENT 2

REPORT OF THE APPEAL PANEL

IN THE MATTER OF

[redacted] ARAVIND REDDY TARUGU

Yuan Chang, MD
Distinguished Professor & American Cancer Society Professor of Pathology

Mary Ganguli, MD
Professor of Psychiatry

Penelope A. Morel, MD
Professor of Immunology

Yoel Sadovsky, MD (Appeal Panel Chair)
Distinguished Professor of Obstetrics, Gynecology & Reproductive Services

John V. Williams, MD
Professor of Pediatrics

March xx, 2018

Introduction

This matter has its origin in a joint Inquiry to assess allegations regarding publications of the Respondent [redacted] Mr. Aravind Reddy Tarugu, Research Associate, Department of Medicine. The Inquiry was started on January 5, 2016 and involved both the University of Pittsburgh and the VA Pittsburgh Healthcare System (VAPHS), with the University serving as the lead institution at the request of VAPHS. The Inquiry Panel recommended the matter be closed without further investigation and the University accepted the recommendation. However, VAPHS asked that an Investigation be conducted. The University agreed to lead a joint Investigation. An Investigative Board considered this matter and based upon their report School of Medicine Assistant Dean for Faculty Affairs Saleem Khan, PhD, found that Mr. Aravind Reddy Tarugu had committed research misconduct through falsification by publishing the same image twice (as mirror images), but with different descriptions. [redacted]

The publication containing the alleged falsification is:

> Lakshmi SP, Reddy AT, Zhang Y, Sciurba FC, Mallampalli RK, Duncan SR, and Reddy RC. *The Journal of Biological Chemistry* 289(10): 6383-6393 (2014). "Down-regulated Peroxisome Proliferator-Activated Receptor γ (PPARγ) in lung epithelial cells promotes a PPARγ agonist-reversible phenotype in chronic obstructive pulmonary disease (COPD)".

On November 27, 2017 Mr. Paul Thaler, on behalf of the Respondent, filed an appeal of these findings and asked that the findings be reversed. On February 9, 2018 the members of the Appeal Panel convened with Dr. Craig Wilcox, Research Integrity Officer of the University of Pittsburgh and Dr. Mara Horwitz, Associate Research Integrity Officer, to discuss the charge to the Appeal Panel and procedures relevant to that charge. The Appeal Panel met again on March 16, 2018.

Grounds for the Appeal of the Finding of Research Misconduct

The University of Pittsburgh 2008 Research Integrity Policy (Policy #11-01-01) states that grounds for an appeal are limited to (1) failure to follow appropriate procedures; (2) insufficiency of evidence; or (3) arbitrary and capricious decision making.

The relevant grounds of the Respondent' appeal are as follows: (1) There was a failure to follow standard procedures resulting in (a) failure to secure evidence, (b) failure to provide proper notification to the Respondent prior to the appearance of the witness, and (c) failure to provide for impartial committees during the Inquiry and Investigation. (2) The research misconduct [redacted] not demonstrated by a preponderance of the evidence, and the Respondent' conduct of research is not a "significant departure from accepted practices" and therefore can constitute neither research misconduct [redacted].

Appeal Panel Activity

The Appeal Panel convened on February 9, 2018 for initial discussion and to receive evidence considered by the Investigative Board. The Panel also received records of the investigative process, the transcript of the Investigative Board hearing, and correspondence in the record including the June 5, 2017 response from Mr. Thaler and the Respondent regarding the draft investigative report, the August 2, 2017 additional comments from Mr. Thaler on behalf of the Respondent, the November 27, 2017

communication of the grounds for appeal, and a January 31, 2018 communication from Mr. Thaler regarding supplemental information pertaining to a desktop computer discussed in the report of the Investigative Board. The grounds for an appeal as described by the University of Pittsburgh Research Integrity Policy (cited above) were reviewed. The appeal filed on behalf of the Responden[redacted] was discussed and each of the specific grounds for an appeal of the findings of Research Misconduct was discussed.

Following the February 9, 2018 meeting the Appeal Panel considered the record and the documents received in support of the appeal and then reconvened on March 16, 2018.

Appeal Panel Recommendations

The Appeal Panel agreed unanimously to recommend reversal of the finding of Research Misconduct on the part of Mr. Aravind Reddy Tarugu.

[redacted]

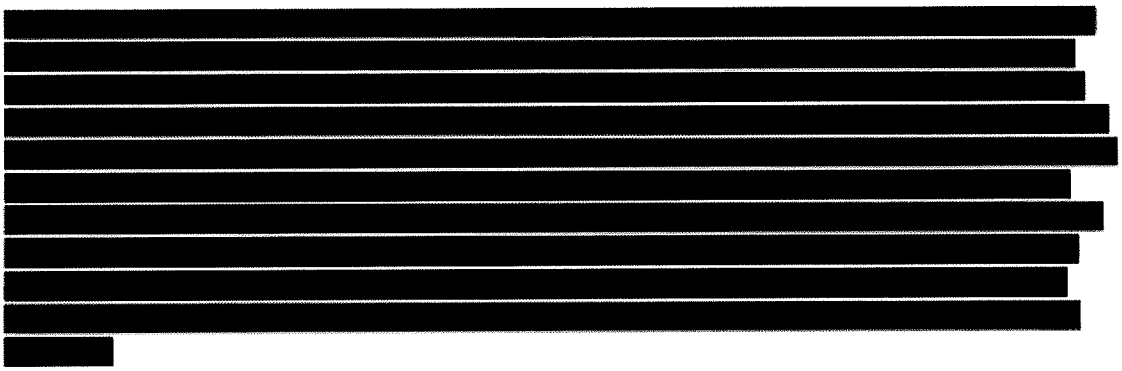
Appeal Panel Advice Regarding the Grounds for the Appeal

Research Misconduct Finding

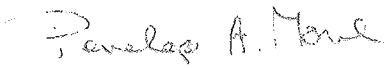
The Panel offers the following comments regarding the claims made by the Responden[redacted] in the appeal of the research misconduct finding:

> *The misconduct is based solely on the apparent match of the images. The issue of the bands being identical is not conclusively resolved. There is insufficient evidence of intentionality.*
>
> Agreed. There was disagreement between the two experts who examined the image evidence, and that disagreement was not fully addressed and resolved by the Investigative Board. [redacted] The Panel also determined that intention was not proven by a preponderance of evidence, including a lack of proof for purposeful image manipulation. The Panel also noted the lack of a motive, as the figure panel at the center of the investigation was not essential for the paper's conclusions.

> *The investigation was infected with irregularities. Witness procedures were not followed.*
>
> Agreed with qualifications. The sequestration process was deficient. The Panel felt the University made the appropriate attempts to sequester the relevant information but the usual procedures were hampered by issues that arose due to VAPHS imposed data security rules and technical capabilities. [redacted]



Signature Page

Panel Member	Date
	March 28, 2018
Yuan Chang, MD Distinguished Professor & American Cancer Society Professor of Pathology	
	March 29, 2018
Mary Ganguli, MD Professor of Psychiatry	
	March 28, 2018
Penelope A. Morel, MD Professor of Immunology	
	March 26, 2018
Yoel Sadovsky, MD (Appeal Panel Chair) Distinguished Professor of Obstetrics, Gynecology & Reproductive Sciences	
	March 25, 2018
John V. Williams, MD Professor of Pediatrics	

ATTACHMENT 3

Price Research Integrity Consultant Experts

Alan R. Price, Ph.D.

21704 Sierra Trail, Lago Vista, Texas

Cell phone 512-483-1574

Email resmiscon@researchmisconductconsultant.com

Paul Thaler and Karen S. Karas, Counsels
Cohen Seglias Pallas Greenhall & Furman PC
1828 L Street, NW, Suite 705
Washington, DC 20036

August 10, 2018

RE: Opinion on DVA-required notice to *JBC* Editor by Aravind Reddy on 2014 paper finding

You requested my expert opinion, regarding a Department of Veterans Affairs (DVA) remedial action plan for Mr. Aravind Reddy ("Mr. Reddy"), requiring notification of the *Journal of Biological Chemistry* (*JBC*), following a University of Pittsburgh (UPitt) Appeal Panel Report of March 27, 2018, and the UPitt Provost's acceptance letter of April 4, 2018, reversing the finding of an earlier joint UPitt/Veterans Administration Pittsburgh Health Care System (VAPitt) investigation of research involving two western blot β-actin bands, published in Figure 5E in *JBC* 289(10):6383 (2014), with technical assistance from Mr. Reddy as the second author. You previously provided me with the UPitt/VAPitt inquiry and investigation reports and exhibits, which I reviewed in October 2017 for my expert opinion on the case process and initial findings.

I understand that, given the recent reversal of the UPitt findings, UPitt officials also reversed the proposed sanction that would have required Mr. Reddy to request *JBC* retract this publication. However, I understand that DVA has affirmed its own finding against Mr. Reddy, and DVA is requiring him to notify *JBC* about that finding for this publication.

As background, I was a faculty member at the University of Michigan from 1970-1987. I retired from the federal Office of Research Integrity (ORI) after 17 years in 2006, serving the last six years as ORI Associate Director for Investigative Oversight. We handled over 3,000 allegations/queries and over 700 formal inquiries/investigations and made over 175 findings of scientific/research misconduct. During my last decade there, I reviewed and handled all of the allegations received from individuals, institutions, and the National Institutes of Health (NIH). We also reviewed and analyzed all of the reports of inquiries/investigations by universities, hospitals, and other research institutions funded by NIH research grants. I believe that no one person has seen, handled, and resolved more allegations, investigations, and oversight cases of scientific/research misconduct than I have. I also communicated with many journal editors, including at *JBC*, on correction or retraction of publications with allegedly falsified images or other data. I was a biochemist and member of FASEB's Biochemistry Society which hosts *JBC*. For the past twelve years, I have been consulting formally with individuals and institutional officials on such matters, as Price Research Integrity Consultant Experts (P.R.I.C.E.).

DVA notice to Mr. Reddy on notifying the *JBC* of the DVA's finding

On July 9, 2018, the Research Integrity Officer (RIO) and Associate Chief of Staff, Research and Development at VAPitt, sent a memo to Mr. Reddy, outlining a remedial action plan for him to follow, specifically stating:

> You must notify the Journal of Biological Chemistry (JBC) of the VA's finding of research misconduct regarding the data published in the JBC 289(10):6383-6393 (2014)... [which requires] each author is required to provide details of the finding.

My opinion on Mr. Reddy notifying the *JBC* of DVA's finding of research misconduct

In notifying the Editor of *JBC* of the finding as required in the remedial action plan memo by the VAPitt RIO, I believe Mr. Reddy should quote the research misconduct finding in the joint UPitt/VAPitt investigation committee report:

> **Statement of Findings - Research Misconduct**
> We find the allegation stated in the charge to our committee to be true. In Figure 5E of the JBC 2014 paper referenced above, the first band in the left panel in the β-actin blot (lane 6) is indeed sufficiently identical to the first band in the right panel of the pMSK1 blot (when reflected across a vertical line) to support the allegation...
>
> We find that the creation and publication of these two images, one derived from the other through digital manipulation, is falsification and that the falsification was intentional. The evidence of intent is that the same image was chosen twice (an action very unlikely to occur by accident) and a mirror image of one of the images was created (a second action very unlikely to occur by accident). We find research misconduct...

I believe Mr. Reddy should also quote the statement in the VAPitt RIO's July 9, 2018, remedial action memo above, that the Network Director of VA Healthcare-VISN 4 had concluded that he:

> ... committed research misconduct by falsifying data with 2 blots being the mirror image of each other and represented as different blots in a publication.

In addition, I believe Mr. Reddy should quote the conclusions in the March 27, 2018, UPitt Appeal Panel Report (p. 3), with which I very strongly concur:

> The Appeal Panel agreed unanimously to recommend reversal of the finding of Research Misconduct on the part of Mr. Aravind Reddy Tarugu....
>
> The Panel offers the following comments regarding the claims made by the Respondent in the appeal of the research misconduct finding:

The misconduct is based solely on the apparent match of the images. The issue of the bands being identical is not conclusively resolved. There is insufficient evidence of intentionality.

Agreed. There was disagreement between the two experts who examined the image evidence, and that disagreement was not fully addressed and resolved by the Investigative Board.... The Panel also determined that intention was not proven by a preponderance of evidence, including a lack of proof for purposeful image manipulation. The Panel also noted the lack of a motive, as the figure panel at the center of the investigation was not essential for the paper's conclusions.

The investigation was infected with irregularities. Witness procedures were not followed.

Agreed with qualifications. The sequestration process was deficient. The Panel felt the University made the appropriate attempts to sequester the relevant information but the usual procedures were hampered by issues that arose due to VAPHS imposed data security rules and technical capabilities.

My opinion on the lack of justification for a retraction of the 2014 *JBC* paper

Based on my almost three decades of reviewing over 800 investigation reports, I strongly agree with the Appeal Panel that intentional falsification by Mr. Reddy, with manipulation of the control blot data in Figure 5E, was not proven by a preponderance of evidence. Therefore, as the Appeal Panel concluded, a research misconduct finding against Mr. Reddy is unwarranted.

I recommend that Mr. Reddy notify the Editor of *JBC*, about the VAPitt finding, supplying the above information, and arguing strongly, as I have, that no retraction of the 2014 *JBC* paper is warranted.

Sincerely,

Alan R. Price, Ph.D.
Price Research Integrity Consultant Experts
Former Associate Director of the U.S. Office of Research Integrity

ATTACHMENT 4

TO: **Paul Thaler, Karen Karas**

FROM: **John Dahlberg, PhD**

DATE: **August 10, 2018**

SUBJECT: Request of the Department of Veterans Affairs (VA) that Mr. Aravind Reddy notify Journal of Biological Chemistry (JBC) regarding the finding made against him in the matter of JBC paper 289:10, pp. 6383–6393, March 7, 2014 ("the JBC paper")

I have been asked to opine on the appropriateness of the VA's adherence to the University of Pittsburgh Investigative Committee's recommendation that the referenced published paper be retracted by one of the co-authors. By way of background, after twenty five years as a bench scientist, I jointed the Office of Research Integrity (ORI) within the Department of Health and Human Services, where I spent an additional twenty three years as a scientist/investigator in the Division of Investigative Oversight (DIO). From 2006-2013 I was the Director of DIO, and from 2013-2015 I served as the Deputy Director of ORI, at which time I retired from federal service. While at ORI, I evaluated thousands of allegations of possible research misconduct, and conducted oversight review and analyzed hundreds of inquiries and investigations of cases of alleged research misconduct involving individuals and institutions from all regions of the United States.

One of the principal objectives of ORI was to help ensure the accuracy of the scientific literature, and when we concurred with institutional findings of research misconduct and made separate findings of misconduct on behalf of the federal government, we typically requested correction or retraction of articles when the scope of the findings warranted such an action. Of course, ORI realized that neither we nor the respondents who agreed to voluntary settlements with ORI could demand that editors retract a paper, as that determination more appropriately rests with the journal's editors who are more qualified to judge the impact of the misconduct on the overall accuracy of the published manuscript and the impact of the fabrications, falsifications, and/or plagiarism on the scientific community.

I have reviewed the single issue on which the VA determined, based on the Investigative Report made by the University of Pittsburgh and VAPHS's joint investigation led by the University, that Mr. Reddy had committed research misconduct, thereby warranting, in its view, retraction of the referenced JBC paper. I note that the alleged infraction involves a claim that two bands in Figure 5E appear to be the same.[1] After careful consideration of the appeal to the Provost, the University of Pittsburgh found that there was insufficient evidence to prove that these two bands were identical (or as ORI would phrase it, had a common origin), and did not find that Mr. Reddy had committed research misconduct.

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[1] "the first band in the left panel in the β-actin blot appears to be the same as the first band in the right panel of the pMSK1 blot, if one or the other was reflected across a vertical reference line" (University of Pittsburgh Investigation Report, page 5)

ORI dealt with similar claims on many occasions and nearly always determined that forensic comparison of two bands might show that they appeared very similar or perhaps identical, but that in the absence of additional evidence of possible misconduct, it would be inappropriate to make a finding of research misconduct based on a single possibly duplicated band. In this case, the JBC paper contains multiple figures with western blots containing approximately 150 lanes of blots with approximately 650 separate bands. None of the remaining bands were alleged to have been duplicated. ORI **never** made findings of research misconduct based on such a flimsy evidentiary basis.

The University Appeal Panel also noted in their response to the UP-investigation report that the figure in question was of marginal significance. The panel also noted that because the questioned panel was not essential to the paper's conclusions, it could not determine that anyone would have had a motive to falsify the figure.

It should also be noted that the questioned images are control blots, and that the research record described two separate experiments containing corresponding blot images.

For these reasons, I respectfully recommend that the editors of JBC agree with UP and Mr. Aravind Reddy to not retract the referenced paper. I am proud of the efforts made by ORI during my tenure to be cognizant of the adverse consequences of a finding of research misconduct to the reputation of a scientist; in cases such as this ORI would not have concurred with the VA's findings or recommendations.

John Dahlberg